

# Cell proliferation and death in the brain of active and hibernating frogs

Silvia Cerri,<sup>1</sup> Giovanni Bottiroli,<sup>3</sup> Maria Grazia Bottone,<sup>1,3</sup> Sergio Barni<sup>2,3</sup> and Graziella Bernocchi<sup>1,3</sup>

<sup>1</sup>Dipartimento di Biologia Animale, Laboratorio di Biologia Cellulare e Neurobiologia, <sup>2</sup>Dipartimento di Biologia Animale, Laboratorio di Anatomia Comparata e Citologia and <sup>3</sup>Istituto di Genetica Molecolare del CNR, Sezione di Istochimica e Citometria, Università di Pavia, Italy

## Abstract

'Binomial' cell proliferation and cell death have been studied in only a few non-mammalian vertebrates, such as fish. We thought it of interest to map cell proliferation/apoptosis in the brain of the frog (*Rana esculenta* L.) as this animal species undergoes, during the annual cycle, physiological events that could be associated with central nervous system damage. Therefore, we compared the active period and the deep underground hibernation of the frog. Using western blot analysis for proliferating cell nuclear antigen (PCNA), we revealed a positive 36 kDa band in all samples and found higher optical density values in the hibernating frogs than in active frogs. In both active and hibernating frogs, we found regional differences in PCNA-immunoreactive cells and terminal transferase dUTP nick-end labelling apoptotic cells in the ventricular zones and parenchyma areas of the main encephalon subdivisions. During the active period of the frogs, the highest concentration of PCNA-immunoreactive cells was found in the ventricle dorsal zone of the cerebral hemispheres but only some of the cells were apoptotic. By contrast, the tectal and cerebellar ventricular zones had a small or medium amount of PCNA-immunoreactive cells, respectively, and a higher number of apoptotic cells. During hibernation, an increased PCNA-immunoreactive cell number was observed in both the brain ventricles and parenchyma compared with active frogs. This increase was primarily evident in the lateral ventricles, a region known to be a proliferation 'hot spot'. Although differences existed among the brain areas, a general increase of apoptotic cell death was found in hibernating frogs, with the highest number of apoptotic cells being detected in the parenchyma of the cerebral hemispheres and optic tectum. In particular, the increased number of apoptotic cells in the hibernating frogs compared with active frogs in the parenchyma of these brain areas occurred when cell proliferation was higher in the corresponding ventricular zones. We suggest that the high number of dying cells found in the parenchymal regions of hibernating frogs might provide the stimulus for the ventricular zones to proliferate. Hibernating frogs could utilize an increased cell proliferation in the brain areas as a neuroprotective strategy to face cell death and the onset of neurological damages. Therefore, the hibernator promises to be a valuable model for studying the mechanisms naturally carried out by the central nervous system in order to adapt itself or survive adverse conditions.

**Key words** apoptosis; cell proliferation; frog brain; hibernation and activity.

## Introduction

The process of adult neurogenesis is linked to the birth and maturation of new neurons that add to or replace neurons in existing circuitry under normal conditions. Research on vertebrate models, particularly mammals, has recently received a great deal of attention (for a review see Lindsey & Tropepe, 2006). In the last decade, the central

nervous system (CNS) of teleostean fish has emerged as a prominent model, well-known for its robust neurogenic capacity as largely studied by Zupanc (cited by Lindsey & Tropepe, 2006). Moreover, in the last few years, attention has also been paid to adult neurogenesis in non-mammal vertebrates (Kaslin et al. 2008), including amphibians (Bernocchi et al. 1990; Polenov & Chetverukhin, 1993; Dawley et al. 2000; Margotta et al. 2005; Raucci et al. 2006; Almlí & Wilczynski, 2007; Simmons et al. 2008).

A variety of natural mechanisms influence the rate of adult neurogenesis. For instance, seasonal variation (Alvarez-Buylla & Lois, 1995; Clayton, 1998; Dawley et al. 2000; Nottebohm, 2002a,b; Hansen & Schmidt, 2004; Hoshooley & Sherry, 2004), temperature and photoperiod (Ramirez et al. 1997; Peñafiel et al. 2001), and physical activity

### Correspondence

Prof. Graziella Bernocchi, Dipartimento di Biologia Animale, Laboratorio di Biologia Cellulare e Neurobiologia, Piazza Botta 10, I-27100 Pavia, Italy. T: + 39/0382/986327; F: + 39/0382/986325; E: bern@unipv.it

Accepted for publication 25 April 2009

Article published online 15 June 2009

(Bernocchi, 1985; Bernocchi et al. 1986, 1990; van Praag et al. 1999a,b) all affect cell proliferation and neuronal differentiation. Among the natural mechanisms, hibernation is a fascinating strategy for some species of animals to survive when the environmental temperature is very low and food resources are scarce (Wang & Lee, 1996). It is interesting that successful hibernation requires a variety of regulatory adaptations including those that coordinate the profound metabolic rate depression and others that reorganize cellular activities such as proliferation, differentiation and cell death (Bernocchi, 1985; Wang & Lee, 1996; Storey, 2001; Barni et al. 2002; Storey & Storey, 2004). Hibernating animals survive without neurological damage (Frerichs et al. 1994; Frerichs, 1999) and, experimentally, against neurological insults (Bullard et al. 1960; Frerichs et al. 1994; Frerichs & Hallenbeck, 1998; Frerichs, 1999; Zhou et al. 2001). Interestingly, brains of hibernating mammals are protected against a variety of insults that are detrimental to humans and other non-hibernating species (Drew et al. 2001).

In the maturation of the CNS, neurogenic events are regulated by cell death, which assures the number and distribution of neurons in the brain network (Lossi et al. 1998; Lossi & Merighi, 2003). More generally, a precise balance of cell production and cell death is required to maintain an accurate number of cells in regenerative tissues. In fact, a deficit of apoptosis during CNS development provokes an increase of neurons as well as a structural and functional disorganization in some areas of the brain (Kuida et al. 1996, 1998).

There are at least two reasons that led us to examine cell proliferation and apoptosis in the adult frog more closely. Firstly, we previously described the presence of new cells by means of ( $^3\text{H}$ )thymidine uptake in the adult frog (Bernocchi et al. 1990) but we did not study the fate (survival or death) of the neural cells at that time. Secondly, we found very few ( $^3\text{H}$ )thymidine-labelled cells during hibernation in that study; however, it is now possible to use more sensitive and less difficult markers. Moreover, the study of the CNS in the hibernation state requires particular attention as hibernation induces several physiological changes (Dave et al. 2006) that could be associated with CNS damage. We thus thought it noteworthy to map cell proliferation/apoptosis in the neurogenic areas of the brain in a model of deep hibernation. Interestingly, whereas in mammals short periods of dormancy prevail (Lyman et al. 1982), in the underground hibernating frogs of northern Italy (*Rana esculenta*) the inactive period generally lasts for the entire winter season from December to March (Bernocchi, 1985). For this research, we used proliferating cell nuclear antigen (PCNA) immunohistochemistry and the terminal transferase dUTP nick-end labelling (TUNEL) reaction to study the processes of cell proliferation and cell death in the main subdivisions of the encephalon (forebrain, midbrain and hindbrain). In particular, for each encephalic subdivision, we considered the brain parenchyma and ventricular lining rather than the specific areas/nuclei.

## Materials and methods

### Animals

Adult male frogs (*R. esculenta* L.) of about 6–7 cm in length (legs excluded) and 25–30 g body weight were used. The animals were collected in their natural environment (Pavia, Italy) in June (20–25 °C) during the period of activity and in January (0–5 °C) during underground hibernation. After collection, the frogs ( $n = 12$ , six 'active frogs' and six 'hibernating frogs') were anesthetized by immersing in 0.1% MS222 (tricaine methanesulfonate, Sandoz, Switzerland) and killed by decapitation.

### Sample preparation

In one group ( $n = 6$ , three active frogs and three hibernating frogs), the brains were removed and immediately fixed in Carnoy solution (6 parts absolute ethanol : 3 parts chloroform : 1 part glacial acetic acid) for 12 h, kept in absolute ethanol and acetone, and then embedded in Paraplast X-tra. Sections (8  $\mu\text{m}$  thick) of the whole brains were cut in the sagittal plane and four series of three slides each with (four) adjacent sections were made per animal. A subset of the slides from each animal was then processed for PCNA immunohistochemistry and another for the TUNEL histochemical reaction (as described below). To avoid possible staining differences due to small changes in the procedure, the reactions were carried out simultaneously on slides of active and hibernating frogs. All experimental procedures were in accordance with the European Community's Council Directive (86/609/EEC).

### Western blot

Western blot analyses for PCNA were performed first on crude extracts of frog brain to determine whether the antibody raised in mammals is also specific for frog. Brains from the other group of frogs ( $n = 6$ , three active frogs and three hibernating frogs) were removed after anaesthesia and immediately frozen in liquid nitrogen at  $-196^\circ\text{C}$ . The brains were homogenized with an Ultra-Turrax homogenizer in 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 2 mM EDTA, 0.2% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 25  $\mu\text{g mL}^{-1}$  aprotinin and 25  $\mu\text{g mL}^{-1}$  leupeptin (Sigma, St Louis, MO, USA), and sonicated in sodium dodecyl sulphate loading buffer on ice with 30 s pulses (60 W) and heated for 5 min at 90 °C. Samples were separately electrophoresed in 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis minigel by loading 50  $\mu\text{g}$  protein and transferred to a nitrocellulose filter (BioRad, Hercules, CA, USA) by semi-dry blotting for 1.45 h under a constant current of 70 mA. The membrane was blocked overnight with phosphate-buffered saline (PBS) containing 0.2% Tween 20 and 5% non-fat dry milk. The sheet was then incubated for 1 h with mouse monoclonal antibody against PCNA (Oncogene, Boston, MA, USA) diluted 1 : 2000. The membranes were then washed three times and incubated for 1 h with anti-mouse horseradish peroxidase-conjugated secondary antibody (DAKO, Milan, Italy) diluted 1 : 2000. Visualization of the immunoreactive bands was performed by electrochemiluminescence detection using Hyperfilm Photographic Film (Amersham Life Sciences, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions. Quantification of the immunoreactive band on the film was made by densitometric measurements by means of the 'measure' feature of the Image J program (1.35 h release, NIH, Bethesda, MD,

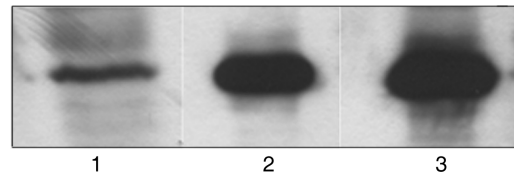
USA). The integrated density values measured on each selected band of the lanes were corrected for the background evaluated on a corresponding area selected out of the lane. Student's *t*-test was used to compare the means of integrated density values.

### Proliferating cell nuclear antigen immunohistochemistry

The paraffin sections were dewaxed in xylene, rehydrated in a decreasing ethanol series, rinsed in distilled water and immunostained as described below. Endogenous peroxidases were suppressed by incubation of sections with 3% H<sub>2</sub>O<sub>2</sub> in 10% methanol in PBS (Sigma) for 7 min. Subsequently, the sections were incubated for 20 min in normal horse serum at room temperature (24 °C) so as to block nonspecific antigen binding sites. Localization of PCNA was achieved by sequentially applying, on the brain sections, a mouse monoclonal anti-PCNA antibody (1 : 600; Oncogene) in PBS overnight in a dark moist chamber, biotinylated secondary antibody (1 : 200, Vector Laboratories, Burlingame, CA, USA) for 30 min and horseradish peroxidase-conjugated avidin-biotin complex (Vector Laboratories) for 30 min at room temperature. Then 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Sigma) with 0.01% H<sub>2</sub>O<sub>2</sub> in Tris-HCl buffer (0.05 M, pH 7.6) was used as chromogen. After each reaction step, sections were washed thoroughly in PBS (two changes, 5 min each). Sections were counterstained with haematoxylin, dehydrated in ethanol, cleared in xylene and mounted in Eukitt (Kindler, Freiburg, Germany). The slides were observed with an Olympus BX51 microscope and the images were recorded with an Olympus Camedia C-5050 digital camera and stored on a PC using Olympus software for processing and printing. For the control staining, some sections were incubated with PBS instead of the primary antibody. No immunoreactivity was present in these conditions.

### Terminal transferase dUTP nick-end labelling histochemical reaction

The reaction was performed using a terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling kit (Roche, Penzberg, Germany). The paraffin sections were dewaxed in xylene, rehydrated in a decreasing ethanol series, rinsed in PBS (Sigma) and incubated with a permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate in PBS) for 8 min at room temperature (24 °C). After rinsing twice in PBS, slides were incubated with 50 µL of TUNEL mixture conjugated with fluorescein isothiocyanate (5 µL terminal deoxynucleotidyl transferase solution and 45 µL label solution) according to the manufacturer's instructions for 3 h at 37 °C. The sections were rinsed in PBS and the cells were counterstained with 0.1% trypan blue in PBS. After washings with PBS, the nuclei were counterstained with 0.1 µg mL<sup>-1</sup> Hoechst 33258 for 5 min and coverslips were mounted in a drop of Mowiol (Calbiochem, Darmstadt, Germany). The slides were observed by fluorescence microscopy with an Olympus BX51 microscope equipped with a 100 W mercury lamp used under the following conditions: 330–385 nm excitation filter, 400 nm dichroic mirror and 420 nm barrier filter for Hoechst 33258, and 450–480 nm excitation filter, 500 nm dichroic mirror and 515 nm barrier filter for fluorescein isothiocyanate. Images were recorded with an Olympus Camedia C-5050 digital camera and stored on a PC using Olympus software for processing and printing. Some sections were incubated without enzyme solution for the control staining. No reactivity was present in these conditions.



**Fig. 1** Western blot analyses of proliferating cell nuclear antigen (PCNA) protein in brain extracts of active and hibernating frogs. In comparison with co-migrating size marker, the anti-PCNA antibody revealed a specific band of about 36 kDa in all samples. The optical density values are higher in hibernating than in active frogs. (1) Size marker, (2) active frogs, (3) hibernating frogs.

### Counting of labelled cells

Sections at the same level were chosen for cell counting in the active and hibernating frogs. Slides were coded for blind counting. PCNA-labelled cells were counted under a light microscope (Leitz Laborlux K) with 20× objective (unless multiple cells overlapped and then a 40× objective was used to confirm the number of labelled cells). PCNA-positive nuclei with clear apoptotic signs (nuclei with clumps of condensed chromatin or nuclei fragments as described in Böhm & Schild, 2003) were excluded. TUNEL-stained nuclei were counted by epifluorescence microscopy (Olympus BX51). For each marker, a total sample of 54 sections (18 per animal) from active frogs and 54 sections (18 per animal) from hibernating frogs was used to count the number of cells in the ventricular zone and parenchyma of the encephalon. We calculated the mean of the counts per section for each animal (individual mean value). As no significant regional differences were found among the three active frogs or the three hibernating frogs (as demonstrated by one-way ANOVA followed by Tukey HSD test), a Student's *t*-test was used for the comparison between the means of the individual mean values (Table 1).

## Results

The anti-PCNA antibody revealed a positive 36 kDa band in all samples. The integrated optical density values were found to be higher in the brains of hibernating than of active frogs ( $1.7 \pm 0.2$  vs  $1.1 \pm 0.1$ ,  $P < 0.01$ ) (Fig. 1). The regional differences of PCNA-immunoreactive (ir) proliferating cells and TUNEL apoptotic cells in the ventricular zones and parenchyma of the main encephalon subdivisions in the active and hibernating frogs are reported below.

### Forebrain

In active frogs, PCNA-labelled cells were located around the ventricles (Table 1), especially in the ventricle dorsal lining (Fig. 2a). In this zone, some cells might be ependymal in nature as they were elongated; these columnar cells often appeared in clusters or pairs. Some labelled nuclei were also present below the ependymal layer. In the ventricle ventrolateral zone, they were small and round in shape (Fig. 2a). A few PCNA-ir cells (Table 1) were scattered in the parenchyma of olfactory bulbs and cerebral hemispheres, and in the thalamic parenchyma,

**Table 1** Count of proliferating cell nuclear antigen (PCNA)- and terminal transferase dUTP nick-end labelling (TUNEL)-positive cells in the brain of active and hibernating frogs

Encephalic subdivisions and areas		Active frogs		Hibernating frogs	
		PCNA-labelled cells	TUNEL-labelled cells	PCNA-labelled cells	TUNEL-labelled cells
Forebrain	Olfactory bulb parenchyma	2.9 ± 0.2	–	6.2 ± 0.2***	15.4 ± 1.2
	Olfactory bulb ventricle	3.9 ± 0.7	–	7.7 ± 0.3***	2.6 ± 0.8
	Cerebral hemisphere parenchyma	1.4 ± 0.2	20.3 ± 2.8	9.8 ± 1.3***	32.0 ± 4.3*
	Ventricle dorsal zone	14.6 ± 5.9	2.8 ± 0.2	42.2 ± 5.0**	2.6 ± 0.2 (NS)
	Ventricle ventrolateral zone	6.3 ± 0.6	3.4 ± 0.5	39.2 ± 3.8***	2.6 ± 0.7 (NS)
	Thalamic parenchyma	1.3 ± 0.3	–	6.0 ± 0.9***	–
	Preoptic area	3.2 ± 0.4	–	18.2 ± 3.4**	4.9 ± 0.4
	Infundibular recess	2.9 ± 0.2	–	17.4 ± 1.3***	9.8 ± 1.7
Midbrain	Tectal layers	1.1 ± 0.2	4.7 ± 0.7	5.8 ± 0.7***	30.1 ± 1.8***
	Tectal ventricle	1.3 ± 0.3	11.7 ± 0.9	6.7 ± 1.8**	5.4 ± 0.8***
Hindbrain	Cerebellum parenchyma	1.1 ± 0.5	8.1 ± 0.5	2.7 ± 0.9 (NS)	24.9 ± 4.2**
	Cerebellum ventricle	7.3 ± 0.7	22.1 ± 1.1	8.1 ± 1.3 (NS)	27.9 ± 5.2 (NS)
	Medulla oblongata parenchyma	3.8 ± 0.5	5.4 ± 0.7	15.2 ± 1.5***	7.3 ± 0.3*
	Medulla oblongata ventricular zone	11.8 ± 0.5	20.0 ± 1.5	11.2 ± 0.4 (NS)	25.3 ± 0.9**

Results are expressed as the mean of the individual mean values (± S.D.) per encephalic area. The individual mean value represents the mean of the counts per sections for each animal (see Materials and methods).

For each marker, the significance between active and hibernating frogs was tested by using Student's *t*-test (*n* = 3).

\**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, NS, not statistically significant; –, lack of labelled cells.

preoptic and infundibular recesses (Fig. 2e). Very few TUNEL-positive cells were located in the ventricle dorsal and ventrolateral ependyma, whereas several TUNEL-labelled cells were found in the cerebral parenchyma (Fig. 2c). On the contrary, no TUNEL-positive cells were seen in the parenchyma and ventricle of olfactory bulbs as well as in the thalamic parenchyma, preoptic area and infundibular recess (Table 1).

In the hibernating frogs (Fig. 2b), the lining of the lateral ventricles appeared thickened and PCNA-ir cells increased in number as compared with active frogs (Table 1). The PCNA-ir cell distribution was similar to that observed in active frogs; some labelled cells were present in the parenchyma. A conspicuous concentration of PCNA-ir cells was also present in the preoptic and infundibular recesses (Table 1, Fig. 2f), whereas some labelled cells were observed in the thalamic parenchyma and olfactory bulb. There was a high number of TUNEL-positive cells in the cerebral parenchyma (Table 1, Fig. 2d), whereas no changes were revealed in the ventricular lining. TUNEL-positive cells were also observed within the lateral ventricles; they could be ascribed to blood cells (Di Terlizzi & Plat, 2006). Some TUNEL-positive cells were present in the remaining encephalic areas except for the thalamic parenchyma (Table 1).

### Midbrain

In the active frogs, the tectal layers and ventricles (Fig. 3a) had a few solitary PCNA-ir cells, whereas some TUNEL-positive cells were observed, mainly in the ventricular lining

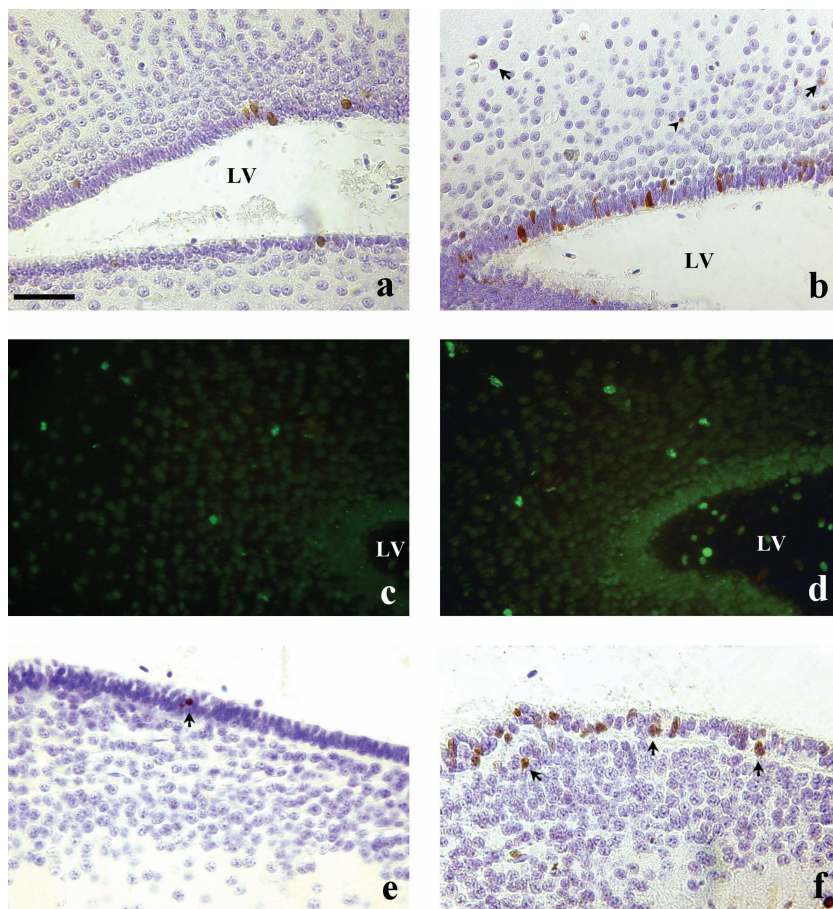
(Table 1, Fig. 3c). Hibernating frogs had some roundish PCNA-ir cells in both the tectal layers and ventricular lining (Table 1, Fig. 3b). Surprisingly, many TUNEL-positive cells were present in the tectal layers (Table 1, Fig. 3d) and some apoptotic cells were found in the tectal ventricle. Inside the optic tectum ventricle there were TUNEL-positive cells, which were probably blood cells.

### Hindbrain

Proceeding caudally into the brainstem, round PCNA-ir cells were located mainly in the ventricular lining of the cerebellum (Fig. 3e) and medulla oblongata (Table 1) of active frogs. Both the ventricular lining of the cerebellum (Fig. 3g) and medulla oblongata had a conspicuous number of TUNEL-positive cells (Table 1). During hibernation, the distribution of PCNA-ir cells in both the parenchyma and ventricular lining of the cerebellum (as shown mainly at the apex, Fig. 3f) and in the ventricular zone of the medulla oblongata was similar to that of the active frogs, whereas more labelled cells were observed in the parenchyma of the medulla oblongata (Table 1). There were several TUNEL-positive cells in the ventricular lining and parenchyma of the cerebellum (Fig. 3h) and in the ventricular lining of the medulla oblongata (Table 1).

### Discussion

Proliferation and cell death appear to be a binomium characterizing adult neurogenesis, similar to that of

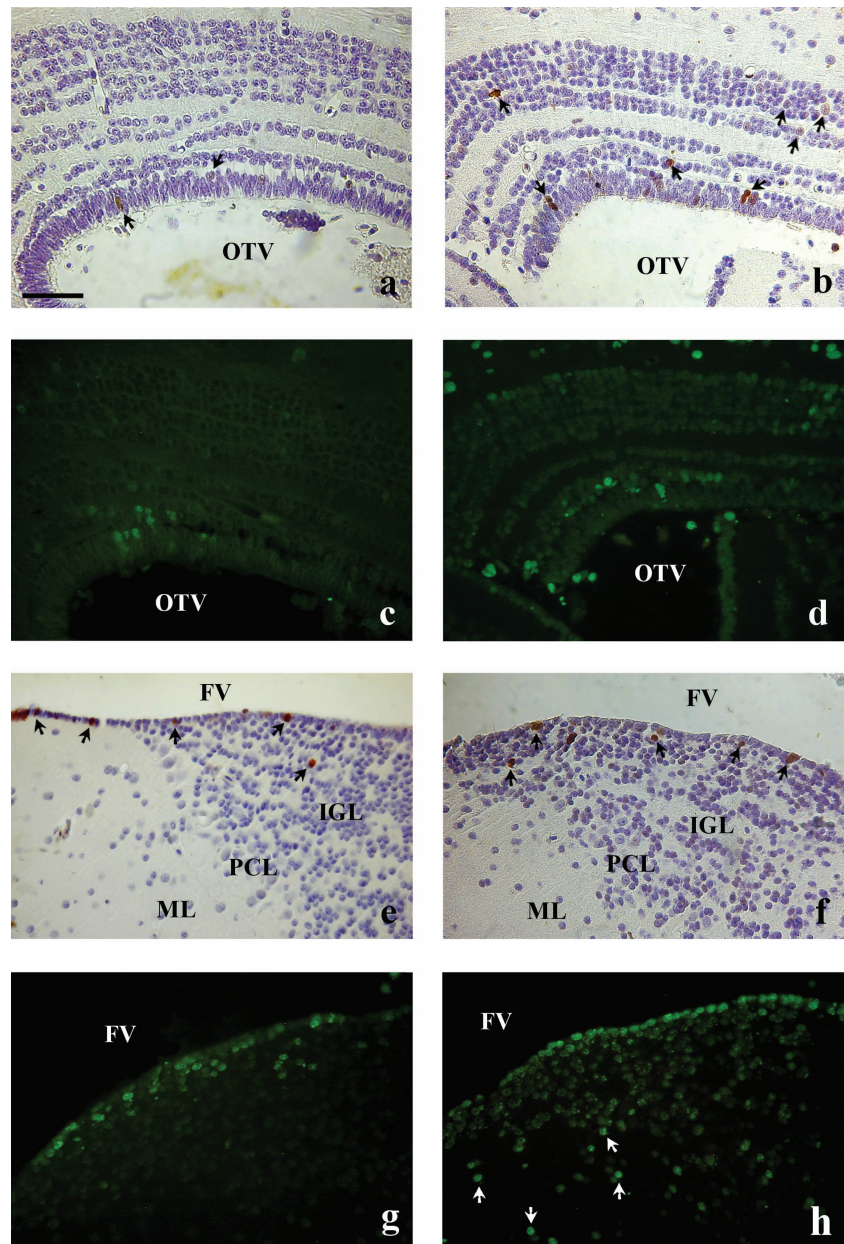


**Fig. 2** Proliferating cell nuclear antigen (PCNA)-labelled cells and terminal transferase dUTP nick-end labelling (TUNEL)-labelled cells in the forebrain of active (a, c, e) and hibernating (b, d, f) adult frogs. In comparison with active frogs (a), a higher number of PCNA-immunoreactive (ir) cells (arrows) is evident in both cerebral hemispheres parenchyma and ventricles of hibernating frogs (b); a PCNA-positive nuclear fragment is shown (b, arrowhead). More numerous TUNEL-positive nuclei are shown in the cerebral parenchyma during hibernation (d) than in the active period (c). In the infundibular recess of hibernating frogs (f) many PCNA-ir cells (arrows) are detectable compared with active frogs (e). LV, lateral ventricle. Scale bar = 50 µm.

normal developmental neurogenesis (Lossi et al. 1998; Lossi & Merighi, 2003). It is generally believed that a correct balance between cell proliferation and apoptosis during development is fundamental to determine the ultimate architecture of tissues and organs. However, a few studies have dealt with neuronal regeneration and apoptotic cell death in neurogenesis of the adult in the non-mammal vertebrates, such as fish (Zupanc, 2006), also after injury (Takeda et al. 2008). It is known that, in some regions of the adult fish brain, as in most subdivisions of the cerebellum, new cells migrate from the ventricular zones to specific target areas but approximately 50% of the young cells undergo apoptotic cell death. Moreover, no reports exist on the cell proliferation and cell death 'binomium' in the adult hibernating vertebrates, despite the great interest in identifying the mechanisms that heterothermic animals use to reduce or prevent an insult to the CNS during hibernation. In this report we studied brain cell proliferation in active and underground (deep) hibernating frogs using PCNA immunohistochemistry and the detection of PCNA protein by western blot analysis. Further, we mapped the apoptotic cell death using the TUNEL reaction. We found changes in the labelling and distribution of proliferating and dying cells within the main brain regions during hibernation.

Both during activity and hibernation, we demonstrated regional differences in the number of PCNA-labelled cells in the encephalon subdivisions of the adult frogs. The marker of cell proliferation PCNA, also called 'cyclin', is a 36 kDa nuclear protein that is required for DNA replication and repair. It is synthesized during the early G1 and S phases of the cell cycle (Hall et al. 1990), is abundant during the S phase and declines during the G2/M phase (Kurki et al. 1986, 1988). Moreover, PCNA labelling, in addition to indicating proliferating activity, is also an indicator of DNA repair activity (Hall et al. 1990). PCNA is an easy, endogenous marker that photographs several phases of the cell cycle at the time when the animal is killed and could then give a wide picture of the cell proliferation in the neurogenic process. A detailed neuroanatomical mapping of cell proliferation in the brain of *R. esculenta* and *R. catesbeiana* has been made during larval development, metamorphosis and adults using PCNA as a marker (Guioli et al. 2004; Margotta et al. 2005; Raucci et al. 2006; Simmons et al. 2008).

During activity, spontaneous proliferation events, as revealed by PCNA labelling, were ongoing, with the heaviest concentration of proliferating cells being present in the cerebral ventricle dorsal zone. In vertebrates, the cerebral ventricles are considered a 'hot spot' or concentrated region



**Fig. 3** Proliferating cell nuclear antigen (PCNA)-labelled cells and terminal transferase dUTP nick-end labelling (TUNEL)-labelled cells in the midbrain and hindbrain of active (a, c, e, g) and hibernating (b, d, f, h) adult frogs. In comparison with the active period (a, c), a greater number of both PCNA- (arrows) and TUNEL-positive nuclei are present in the tectal layers of hibernating frogs (b, d). An increase of PCNA-immunoreactive cells (arrows) is also observed in the tectal ventricle during hibernation (b). The ventricular lining of the cerebellum shows several PCNA- (arrows) and TUNEL-labelled nuclei in both the active (e, g) and hibernating frogs (f, h). Several TUNEL-positive nuclei (arrows) are present in the cerebellum parenchyma of hibernating frogs (h) compared with active frogs (g). OTV, optic tectum ventricle; ML, molecular layer; PCL, Purkinje cell layer; IGL, internal granular layer; FV, fourth ventricle. Scale bar = 50  $\mu$ m.

of proliferating cells (Alvarez-Buylla et al. 1990). Nevertheless, a few PCNA-ir cells were distributed in the parenchyma. The presence of these cells in the parenchymal regions, more notably in the forebrain, has been interpreted to signify that newly formed cells appear to migrate away from the ventricular zone into the parenchyma (Almli & Wilczynski, 2007). However, as only some of the cells of the ventricular lining became apoptotic there, it may be that in general the proliferating cells are integrated into existing circuitry or are able to replace injured cells (Doetsch & Scharff, 2001; Font et al. 2001; Zupanc, 2001; Cayre et al. 2002; Emsley et al. 2005; Lindsey & Tropepe, 2006).

Hibernation is a very intricate and attractive biological event mainly occurring in non-mammalian vertebrates; it also occurs in some mammals and entails different physio-

logical mechanisms. Specific brain regions also remain active during deep torpor (Heller, 1979), when the hibernator's brain temperature can drop close to the freezing point of water and the brain becomes electrically quiescent to surface electroencephalography (Heller & Ruby, 2004). In fact, as a hibernator enters into deep torpor, the cortex falls 'asleep', which may be, via c-fos expression, a result of the activation of the reticular thalamic nucleus that suppresses arousal activity (Bratincsak et al. 2007). During hibernation, the 'biological clock' is working; the profound activity of the choroid plexus, the ependymal cells of the lateral ventricles and third ventricle suggests that the production of the cerebrospinal fluid or some unrecognized function of these cells might be an important factor in maintaining torpor (Bratincsak et al. 2007).

In comparison with the active period, during hibernation the optical density of PCNA in the brain homogenate significantly increased; this increase fits well with the generally higher number of PCNA-ir cells. Cell proliferation occurred in all of the ventricles and PCNA-ir cells were also present in the parenchyma. The increased number of proliferating cells in the lateral ventricle, with respect to the active period, further supports the notion that this region has continued to be a proliferation 'hot spot'. Data obtained here are in contrast with our previous findings (Bernocchi et al. 1990), which showed a very low incidence of ( $^3\text{H}$ )thymidine-labelled nuclei during hibernation. The difference could be explained, on one hand, by the fact that ( $^3\text{H}$ )thymidine labels only the S-phase and, on the other hand, because of the dramatic drop of body metabolism (Storey, 2003), by the possibility that the dose of ( $^3\text{H}$ )thymidine employed and time before killing were inadequate. PCNA immunocytochemistry encompasses these problems. Further, the possibility that PCNA labelling is also an indicator of DNA repair activity (Hall et al. 1990) cannot be minimized if one considers the likelihood of damage or stress in neural cell populations during hibernation (Bullard et al. 1960; Frerichs et al. 1994; Frerichs & Hallenbeck, 1998; Frerichs, 1999; Zhou et al. 2001).

As regards cell death, TUNEL-positive cells were noticed in the brain of active and hibernating frogs in both ventricular zones and parenchyma. During hibernation, although differences exist among the brain areas, a general increase of apoptotic cell death was found. In the parenchyma regions, the highest numbers of apoptotic cells occurred in the cerebrum, tectum and cerebellum. In addition, contrary to what occurred in the forebrain ventricular zones, the fourth ventricle linings had a high number of apoptotic cells but only some proliferating cells.

Our results also raise the question of whether the PCNA- or TUNEL-labelled cells are neurons or glial cells. As the techniques used here did not permit such identification, further analysis with double immunocytochemistry using neural and glial markers is required. However, it should be considered that neurogenesis and gliogenesis both take place in *R. catesbeiana* (Simmons et al. 2008).

In summary, the findings presented here firstly provide a neuroanatomical mapping of cell proliferation and apoptosis in the brain of both active and hibernating frog. Further, they suggest how these processes can be involved in the neuroprotective strategies carried out during hibernation. Intriguingly, we observed that the increased number of apoptotic cells in the hibernating frogs compared with active frogs in the parenchyma of the olfactory bulbs, cerebral hemispheres and optic tectum occurred when cell proliferation was higher in the corresponding ventricular zones. The high number of dying cells in hibernating frogs in the cerebral hemisphere parenchyma with respect to the number of proliferating cells could be a stimulus for the ventricular zones to proliferate, permitting

the animals to survive, without brain damage, the numerous changes in the mechanisms involving the functions of CNS areas and, more generally, of the physiology of the body (Bullard et al. 1960; Frerichs et al. 1994; Frerichs & Hallenbeck, 1998; Frerichs, 1999; Zhou et al. 2001). Therefore, hibernating frogs seem to utilize an increased cell proliferation in the main brain areas as a strategy to face cell death and the onset of neurological damages. The hibernator promises to be a valuable model for studying the mechanisms naturally carried out by the CNS in order to adapt itself or survive adverse conditions.

## References

- Almli LM, Wilczynski W (2007) Regional distribution and migration of proliferating cell populations in the adult brain of *Hyla cinerea* (Anura, Amphibia). *Brain Res* **1159**, 112–118.
- Alvarez-Buylla A, Lois C (1995) Neuronal stem cells in the brain of adult vertebrates. *Stem Cells* **13**, 263–272.
- Alvarez-Buylla A, Theelen M, Nottebohm F (1990) Proliferation 'hot spots' in adult avian ventricular zone reveal radial cell division. *Neuron* **5**, 101–119.
- Barni S, Vaccarone R, Bertone V, Fraschini A, Bernini F, Fenoglio C (2002) Mechanisms of changes to the liver pigmentary component during the annual cycle (activity and hibernation) of *Rana esculenta* L. *J Anat* **200**, 185–194.
- Bernocchi G (1985) Cytochemical variations in Purkinje neuron nuclei of cerebellar areas with different afferent systems in *Rana esculenta*. Comparison between activity and hibernation. *J Hirnforsch* **26**, 659–665.
- Bernocchi G, Barni S, Scherini E (1986) The annual cycle of *Erinaceus europaeus* L. as a model for a further study of cytochemical heterogeneity in Purkinje neuron nuclei. *Neuroscience* **17**, 427–437.
- Bernocchi G, Scherini E, Giacometti S, Mareš V (1990) Premitotic DNA synthesis in the brain of the adult frog (*Rana esculenta*): an autoradiographic  $^3\text{H}$ -thymidine study. *Anat Rec* **228**, 461–470.
- Böhm I, Schild H (2003) Apoptosis: The complex scenario for a silent cell death. *Mol Imaging Biol* **5**, 2–14.
- Bratincsak A, McMullen D, Miyake S, Toth ZE, Hallenbeck JM, Palkovits M (2007) Spatial and temporal activation of brain regions in hibernation: c-fos expression during the hibernation bout in thirteen-lined ground squirrel. *J Comp Neurol* **505**, 443–458.
- Bullard RW, David G, Nichols CT (1960) The mechanisms of hypoxic tolerance in hibernating and non-hibernating mammals. In *Mammalian Hibernation* (eds Lyman CP, Dawe AR), pp. 322–335. Cambridge, MA: Bulletin of the Museum of Comparative Zoology at Harvard College.
- Cayre M, Malaterre J, Scotto-Lomassese S, Strambi C, Strambi A (2002) The common properties of neurogenesis in the adult brain: from invertebrate to vertebrates. *Comp Biochem Physiol B Biochem Mol Biol* **132**, 1–15.
- Clayton NS (1998) Memory and the hippocampus in food-storing birds: a comparative approach. *Neuropharmacology* **37**, 441–452.
- Dave KR, Prado R, Raval AP, Drew KL, Perez-Pinzon MA (2006) The arctic ground squirrel brain is resistant to injury from cardiac arrest during euthermia. *Stroke* **37**, 1261–1265.
- Dawley EM, Fingerlin A, Hwang D, John SS, Stankiewicz CA (2000) Seasonal cell proliferation in the chemosensory epithelium and brain of red-backed salamanders, *Plethodon cinereus*. *Brain Behav Evol* **56**, 1–13.

- Di Terlizzi R, Plat S (2006) The function, composition and analysis of cerebrospinal fluid in companion animals: Part I. Function and composition. *Vet J* **172**, 422–431.
- Doetsch F, Scharff C (2001) Challenges for brain repair: insights from adult neurogenesis in birds and mammals. *Brain Behav Evol* **58**, 306–322.
- Drew KL, Rice ME, Kuhn TB, Smith MA (2001) Neuroprotective adaptations in hibernation: therapeutic implications for ischemia-reperfusion, traumatic brain injury and neurodegenerative diseases. *Free Radic Biol Med* **31**, 563–573.
- Emsley JG, Mitchell BD, Kempermann G, Macklis JD (2005) Adult neurogenesis and repair of the adult CNS with neural progenitors, precursors, and stem cells. *Prog Neurobiol* **75**, 321–341.
- Font E, Desfilis E, Pérez-Cañellas MM, García-Verdugo JM (2001) Neurogenesis and neuronal regeneration in the adult reptilian brain. *Brain Behav Evol* **58**, 276–295.
- Frerichs KU (1999) Neuroprotective strategies in nature – novel clues for the treatment of stroke and trauma. *Acta Neurochir Suppl* **73**, 57–71.
- Frerichs KU, Hallenbeck JM (1998) Hibernation in ground squirrels induces state and species-specific tolerance to hypoxia and aglycemia: an in vitro study in hippocampal slices. *J Cereb Blood Flow Metab* **18**, 168–175.
- Frerichs KU, Kennedy C, Sokoloff L, Hallenbeck JM (1994) Local cerebral blood flow during hibernation, a model of natural tolerance to 'cerebral ischemia'. *J Cereb Blood Flow Metab* **14**, 193–205.
- Guioli S, Pisu MB, Roda E, Bottone M, Boncompagni E, Bernocchi G (2004) Effects of water pollution on the proliferative activity in the developing frog brain. *Ital J Zool* **2**, 89–93.
- Hall PA, Levison DA, Woods AL, et al. (1990) Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: an index of cell proliferation with evidence of deregulated expression in some neoplasms. *J Pathol* **162**, 285–294.
- Hansen A, Schmidt M (2004) Influence of season and environment on adult neurogenesis in the central olfactory pathway of the shore crab, *Carcinus maenas*. *Brain Res* **1025**, 85–97.
- Heller HC (1979) Hibernation: neural aspects. *Annu Rev Physiol* **41**, 305–321.
- Heller HC, Ruby NF (2004) Sleep and circadian rhythms in mammalian torpor. *Annu Rev Physiol* **66**, 275–289.
- Hoshooley JS, Sherry DF (2004) Neuron production, neuron number, and structure size are seasonally stable in the hippocampus of the food-storing black-capped chickadee (*Parus atricapillus*). *Behav Neurosci* **118**, 345–355.
- Kaslin J, Ganz J, Brand M (2008) Proliferation, neurogenesis and regeneration in the non-mammalian vertebrate brain. *Philos Trans R Soc Lond B Biol Sci* **363**, 101–122.
- Kuida K, Zheng TS, Na S, et al. (1996) Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature* **384**, 368–372.
- Kuida K, Haydar TF, Kuan CY, et al. (1998) Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell* **94**, 325–337.
- Kurki P, Vanderlaan M, Dolbeare F, Gray J, Tan EM (1986) Expression of proliferating cell nuclear antigen (PCNA)/cyclin during the cell cycle. *Exp Cell Res* **166**, 209–219.
- Kurki P, Ogata K, Tan EM (1988) Monoclonal antibodies to proliferating cell nuclear antigen (PCNA/cyclin) as probes for proliferating cells by immunofluorescence microscopy and flow cytometry. *J Immunol Meth* **109**, 49–59.
- Lindsey BW, Tropepe V (2006) A comparative framework for understanding the biological principles of adult neurogenesis. *Prog Neurobiol* **80**, 281–307.
- Lossi L, Merighi A (2003) In vivo cellular and molecular mechanisms of neuronal apoptosis in the mammalian CNS. *Prog Neurobiol* **69**, 287–312.
- Lossi L, Zagzag D, Greco MA, Merighi A (1998) Apoptosis of undifferentiated progenitors and granule cell precursors in the post-natal human cerebellar cortex correlates with expression of BCL-2, ICE and CPP-32 proteins. *J Comp Neurol* **399**, 359–372.
- Lyman CP, Willis JS, Malan A, Wang LCH (1982) *Hibernation and Torpor in Mammals and Birds*. New York: Academic Press.
- Margotta V, Morelli A, Caronti B (2005) Expression of PCNA positivity in the brain of normal adult heterothermic vertebrates: further observations. *Ital J Anat Embryol* **110**, 59–74.
- Nottebohm F (2002a) Neuronal replacement in adult brain. *Brain Res Bull* **57**, 737–749.
- Nottebohm F (2002b) Why are some neurons replaced in adult brain? *J Neurosci* **22**, 624–628.
- Peñafiel A, Rivera A, Gutierrez A, Trias S, De la Calle A (2001) Temperature affects adult neurogenesis in the lizard brain. *Int J Dev Biol* **45**, S83–S84.
- Polenov AL, Chetverukhin VK (1993) Ultrastructural radioautographic analysis of neurogenesis in the hypothalamus of the adult frog, *Rana temporaria*, with special reference to physiological regeneration of the preoptic nucleus. II. Types of neuronal cells produced. *Cell Tissue Res* **271**, 351–362.
- Ramirez C, Nacher J, Molowny A, Sanchez-Sanchez F, Irurzun A, Lopez-Garcia C (1997) Photoperiod-temperature and neuroblast proliferation-migration in the adult lizard cortex. *Neuroreport* **8**, 2337–2342.
- Rauci F, Di Fiore MM, Pinelli C, et al. (2006) Proliferative activity in the frog brain: a PCNA-immunohistochemistry analysis. *J Chem Neuroanat* **32**, 127–142.
- Simmons AM, Horowitz SS, Brown RA (2008) Cell proliferation in the forebrain and midbrain of the adult bullfrog, *Rana catesbeiana*. *Brain Behav Evol* **71**, 41–53.
- Storey KB (2001) Turning down the fires of life: Metabolic regulation of hibernation and estivation. In *Molecular Mechanisms of Metabolic Arrest* (ed. Storey KB), pp. 1–21. Oxford: BIOS Scientific Publishers.
- Storey KB (2003) Mammalian hibernation. Transcriptional and translational controls. *Adv Exp Med Biol* **543**, 21–38.
- Storey KB, Storey JM (2004) Metabolic rate depression in animals: Transcriptional and translational controls. *Biol Rev Camb Philos Soc* **79**, 207–233.
- Takeda A, Nakano M, Goris RC, Funakoshi K (2008) Adult neurogenesis with 5-HT expression in lesioned goldfish spinal cord. *Neuroscience* **151**, 1132–1141.
- van Praag H, Christie BR, Sejnowski TJ, Gage FH (1999b) Running enhances neurogenesis, learning, and long-term potentiation in mice. *Proc Natl Acad Sci USA* **96**, 13427–13431.
- van Praag H, Kempermann G, Gage FH (1999a) Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nat Neurosci* **2**, 266–270.
- Wang LCH, Lee TF (1996) Torpor and hibernation in mammals: Metabolic, physiological and biochemical adaptations. In *Handbook of Physiology, Environmental Physiology* (eds Fregley MJ, Blatteis CM), pp. 507–532. Oxford: Oxford University Press.
- Zhou F, Zhu X, Castellani RJ, et al. (2001) Hibernation, a model of neuroprotection. *Am J Pathol* **158**, 2145–2151.
- Zupanc GK (2001) A comparative approach towards the understanding of adult neurogenesis. *Brain Behav Evol* **58**, 246–249.
- Zupanc GK (2006) Neurogenesis and neuronal regeneration in the adult fish brain. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* **192**, 649–670.